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**14. ABSTRACT**

The objectives of this proposal are to test hypotheses: 1) WIF1 IgG fusion protein has an additive or synergistic effect with anti-VEGF therapy to inhibit tumor growth and metastasis; and 2) NRP2 is a wnt target gene and predicts prostate cancer progression. WIF1 IgG fusion protein was produced, but the stability of first fusion protein was poor. Experiments for modification of IgG-WIF1 fusion construct for improving the stability of IgG-WIF1 are in progress. Proposed animal experiments are approved by the UCI IACUC and orthotopic prostate cancer models are established. Chromatin immunoprecipitation (ChIP), luciferase assay, mRNA expression, and bioinformatics analyses demonstrates that NRP2 is a Wnt target gene. NRP2 antibody was validated using tissue array slides.

**15. SUBJECT TERMS**
Neuropilin-2, Wnt signaling, Castration-resistant prostate cancer.

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## Introduction

Anti-Vascular Endothelial Factor (VEGF) based anti-angiogenesis therapy is becoming one of major approaches for treatment of several advanced and metastatic cancers, including castration-resistant prostate cancer (CRPC) [1]. However, development of resistance to anti-VEGF therapy, in particular to Bevacizumab treatment, appears to be very common and inevitable [2]. Currently the underlying mechanisms for Bevacizumab resistance are largely unknown.

Neuropilin-2 (NRP2) and c-Met are co-receptors with each other and with VEGF receptors [3, 4]. Accumulating studies have implicated that both NRP2 and c-Met play important roles in tumor progression and metastasis and are involved in angiogenesis [3, 4]. Based on our preliminary bioinformatics analysis and experiments, we hypothesize that Bevacizumab treatment results in up-regulation of NRP2 and c-Met expression, leading to resistance to Bevacizumab and tumor progression. C-Met is a known Wnt target gene [5]. We also hypothesize that NRP2 is a Wnt target gene, and that expression of c-Met and NRP2 can be down-regulated by a secreted Wnt inhibitor [i.e. Wnt inhibitory factor-1 (WIF-1)]. Therefore, it is possible that WIF-1, in combination with Bevacizumab, can improve the efficacy of Bevacizumab therapy in CPRC and overcome the resistance to Bevcizumab.

To test these hypotheses, three specific aims are proposed as follows: Aim 1: To determine whether WIF1 recombinant protein has an additive or synergistic effect with anti-VEGF therapy to inhibit tumor growth and metastasis in clinically relevant orthotopic models. Aim 2: To determine whether NRP2 is a transcriptional target of canonical Wnt/TCF signaling and contribute to the combined effect of WIF1 and Bevacizumab on cell proliferation, migration and invasion. Aim 3: To determine whether the expression of NRP2 alone or in combination with VEGF receptors or c-Met will be associated with MVD and WIF1 expression, and will be predictive for clinical and biochemical progression-free survivals of PCa patents, as well as for development of metastasis.

The first stage of this proposal is 1) to determine whether NRP2 is transcriptionally regulated by the Wnt pathway, and 2) to produce a recombined WIF-1 protein (i.e. WIF-1 human IgG fusion protein) that can be used as a therapeutic agent for inhibiting Wnt signaling in prostate tumors in animal models and for combined therapy with Bevacizumab.

The second stage of this proposal is 3) to determine the efficacy of WIF-hIgG alone or in combination with Bevcizumab for treatment of CRPC in xenograft mouse models, and 4) to determine whether NRP2 is at least in part required for WIF-hIgG and Bevacizumab mediated inhibition of cell growth, migration and invasion.

The third stage of this proposal is 5) to determine whether NRP2 alone or in combination with VEGF receptors or c-Met is an independent predictor for prostate cancer biochemical recurrence or metastasis.

During the past year, we are on the first stage of this proposal. Due to the technique challenge and the time to hire an experienced post-doctoral fellow for this work, we are not yet successful to produce a WIF-human IgG fusion protein that is stable and useful for preclinical treatment of prostate cancer in xenograft models. However, we have made significant progress in providing evidence that NRP2 is a Wnt target gene and down-regulated by WIF-1. We described our negative and positive findings according to our Statement of Work as follows.

**BODY:**

**Task 1. To determine whether WIF1 recombinant protein has an additive or synergistic effect with anti-VEGF therapy to inhibit tumor growth and metastasis in a clinically relevant orthotopic model.**

**1a.** To write animal protocol and get it approval, test stability and activity of recombinant WIF1 protein and anti-VEGF antibody (1 to 4 months).

(1) Animal protocol was approved by the UCI IACUC (protocol number 2011-2938).

(2) We conducted 1L transient transfection of the recombinant plasmid containing WIF-1-hIgG protein into CHO suspension cells followed by one-step affinity purification using HiTrapTM Mabselect 5ml HP. The target protein was eluted in the gradient of 50mM citrate (pH3.0) followed by buffer exchange. In addition, the second-step purification of WIF-1-hIgG protein was performed by using superdex 200 XK/16 based on one-step affinity purification. This protein samples were then analyzed by SDS-PAGE. The result is shown in Figure 1&2. Two major protein bands including WIF-1-hIgG protein (MW from order information: ~70 kDa) with the desired molecular weight and a ~35 kDa protein that may be the degradation product of the target protein were detected by SDS-PAGE analysis. According to Figure 1&2, the target protein (about 70kDa) cannot be separated by Superdex 200 gel filtration chromatography. The stability of WIF-IgG fusion protein was not ideal. A new post-doctoral fellow (Noriko Yokoyama) with a strong background in molecular biology and Wnt signaling was recruited for continuing this task.

**The first step purification**

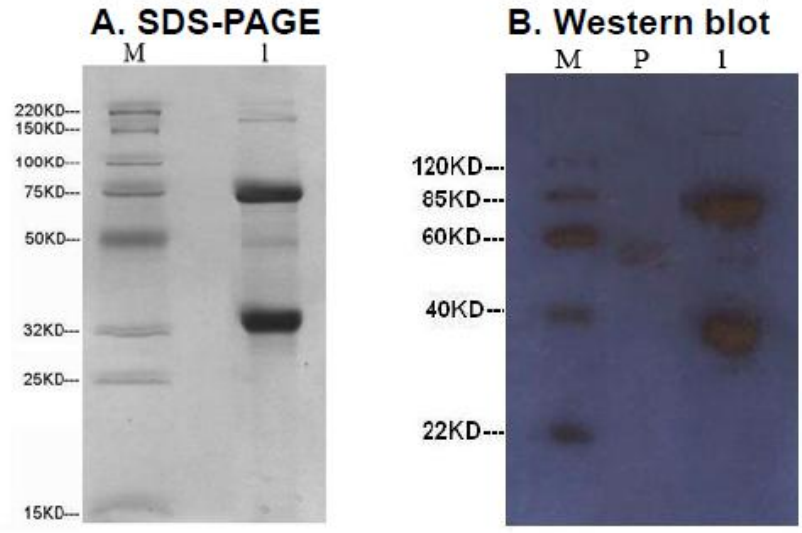



**Figure 1,** SDS-PAGE and Western blot analysis of 97320
**Lane M**: Protein marker **Lane 1**: Purified protein
**Lane P:** Human IgG1, Kappa (Sigma, Cat.No:I5154), as positive control.

**The second step purification**

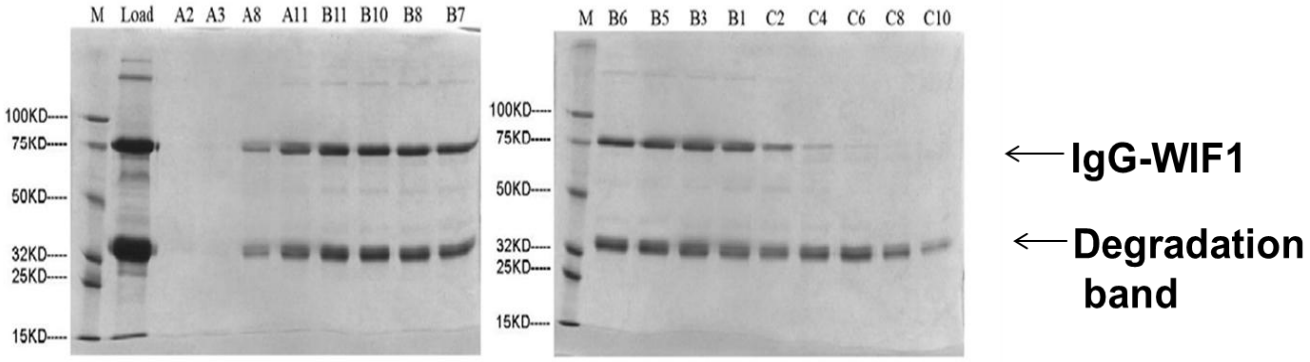



**Figure 2** Purified IgG-WIF fusion protein shows a degraded band around 33 kDa. Lane M: Protein marker Lane load: one-step purified protein Lane A2-C10: purified protein polished by Superdex 200

**1b.** To exmaine stability and pharmacokinetics of recombinant WIF1 protein and anti-VEGF antibody in vivo in mice (4 to 6 months).

We are unable to perform PK study at this stage, due to the stability issue of WIF1 protein.

**1c.** To establish C4-2B and PC3 orthotopic prostate cancer models. A total of 360 C4-2B or PC3 tumor bearing mice will be randomly treated with different doses of WIF hIg Fc, anti-VEGF antibody B20-4.1.1 alone or in combination; Monitoring tumor growth by Xenogen machine and by serum PSA (for c4-2B tumors); Weighting tumor wet weight, documentation organ and tumor status, and harvesting tissues; Performing necropsy and histology (6 to 18 months)

(1) Establishment of C4-2B and PC3 stable cell lines with overexpression of Luciferase gene for *in vivo* imaging of prostate cancer cell growth and metastasis in orthotopic xenograft models. Figure 3 shows that C4-2B-Luc and PC-3-Luc stable clones were selected by G418. C4-2B and PC3 with strongest expression of Luciferase gene will be used for further imaging study.

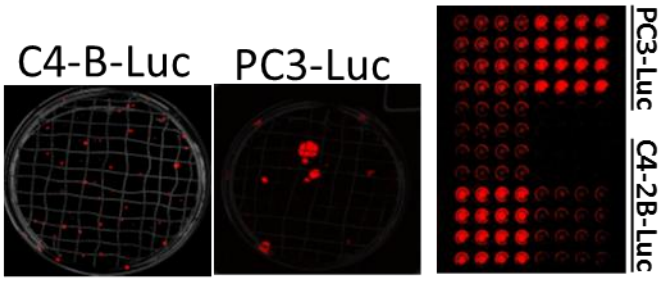


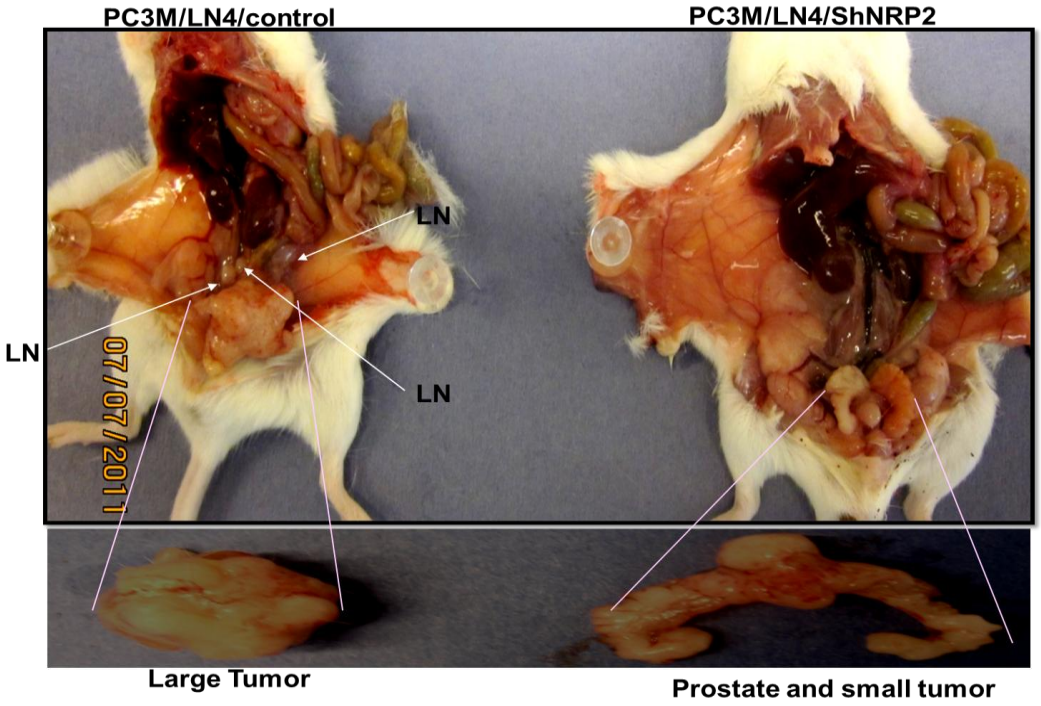

**Figure 3.** C4-2B-Luc and PC3-Luc stable clones exhibit a bioluminescent activity detected by Xenogen machine.

(2) Establishment of the technique for prostatic injection of prostate cancer cells and orthotopic prostate cancer xenograft models.

PC3M/LN4/PcDNA3 and PC3M/ShNRP2 cells were injected into ventral lobe of the prostate of SCID mice. After four weeks, mice were sacrificed and tumor wet weight and lymph node metastasis were examined. Figure 4 shows that stable knock-down of NRP2 expression in PC3M/LN4 cells by short-hairpin RNA results in a decrease in tumor growth and lymph node metastasis (**Figure 4A**). Figure 4B shows that

PC3M/LN4/control cells strongly expressing NRP2 accumulated in the cortex of a lymph node from a xeografted mouse.

**A**




**B**

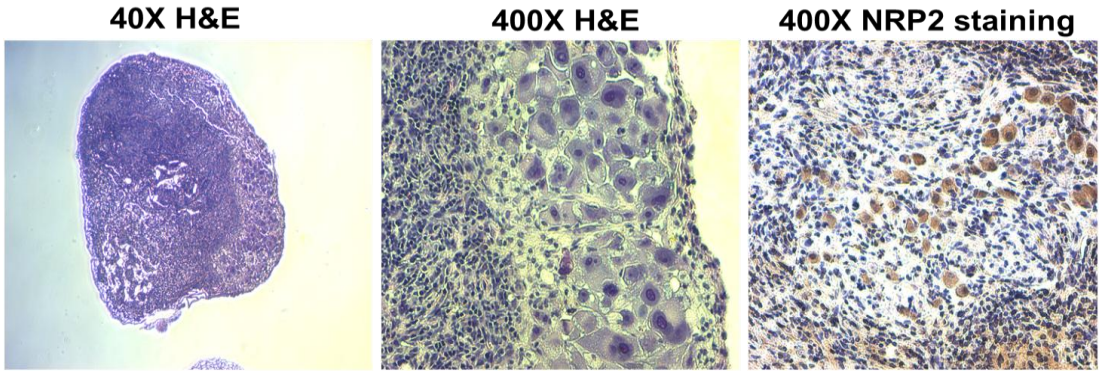



**Figure 4A.** Prostate cancer orthotopic model in mice. **Figure 4B,** Pathological analysis of lymph node metastasis.

**Task 2. To determine whether NRP2 is a transcriptional target of canonical Wnt/TCF signaling and contribute to the combined effect of WIF1 and bevacizumab on cell proliferation, migration and invasion.**

**2a.** To make NRP2 promoter contrstucts and study the effect of Wnt stimulation and inhibition on the activity of NRP2 promoters (months 1-8).

(1) Wnt 3a increase NRP2 mRNA expression and promoter activity. Using real-time RT-PCR, Figure 5A shows that treatment of C4-2B with 100nM recombinant Wnt 3a protein resulted in an about 1.6 fold increase of NRP2 mRNA ($P<0.05$). We next examined the effect of Wnt stimulation on the promoter activity of NRP2. The 3kb

sequence immediately upstream the transcription start site of the NRP2 gene coding regions was cloned into the PGL4 Luc2 luciferase reporter vector. C4-2B cells were then co-transfected with the NRP2/PGL4 luc2 reporter and pRL-CMV, which encodes Renilla luciferase to allow for transfection normalization. After transfection, cells were treated with L cell control and L cell Wnt 3a conditioned medium for 24 hours. Dual luciferase activities of these treated cells were measured under basic conditions. All experiments were repeated at least three times, and all samples were performed in quadruplicate. Figure 5A shows that Wnt3a stimulation increase the NRP2 activity by 3.5 fold (Student t test, $P<0.001$)

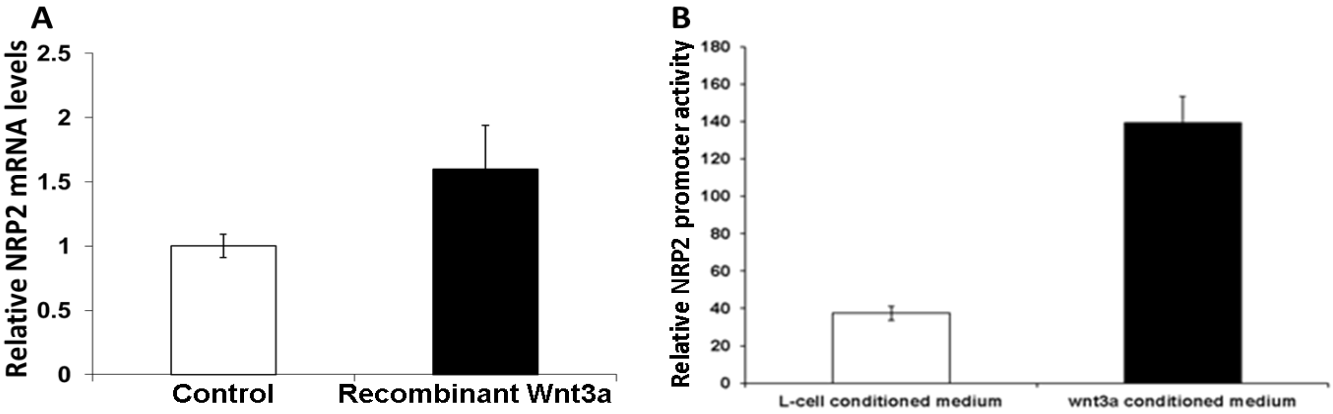



Figure 5. The effect of Wnt3a treatment on the mRNA expression (A) and the promoter activity of NRP2.

(2) Androgen and Wnt stimulation co-regulate the mRNA expression of Wnt transcriptional factors TCF4 and LEF1 and NRP2. To determine whether androgen will affect Wnt signaling and NRP2 expression, we cultured LNCaP cell under charcoal stripped serum conditions and treatment the cells with Wnt 3a and 5 a conditioned medium and 10 nM synthetic androgen R1881. Figure 6 shows that deprivation of androgen in culture medium increased the mRNA expression of TCF4, LEF1 and NRP2. NRP2 mRNA expression was also increased by Wnt 3A and 5A conditioned medium stimulation (CM) compared to control L cell conditioned medium. However, lower levels of TCF4 and LEF1mRNA were restored by addition of synthetic androgen, while the NRP2 mRNA level remained unchanged. These results suggested that androgen deprivation directly regulates the expression of TGF4 and LEF1 leading to indirect up-regulation of NRP2. NRP2 may be directly regulated by TCF4 and LEF1.

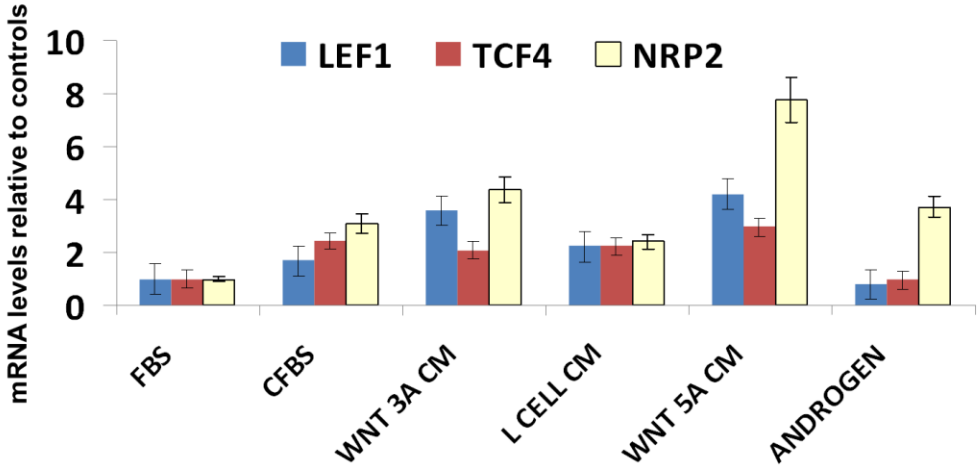



**Figure 6** The effect of Wnt and androgen signaling on the expression of NRP2.

(3) Inhibition of Wnt signaling by ectopic expression of a secreted Wnt inhibitor, Wnt inhibitory factor-1 (WIF1), decreased the protein levels of NRP2 in CRPC cell lines PC3 and C4-2B. We further performed Western blotting analysis of NRP2 protein expression in WIF-1 overexpressing CRPC cell lines. Compared to vector control, WIF-1 expression completely or highly potently inhibited the protein expression of NRP2 in both C4-2B and PC3 cells (Figure 7).

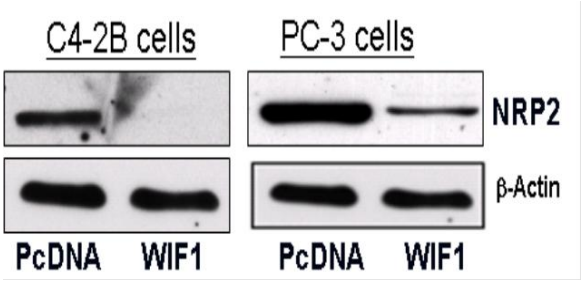



**Figure 7.** ectopic expression of WIF1 down-regulates the expression of NRP2

(4) WIF1 mRNA levels are inversely correlated with NRP2 mRNA levels in prostate and prostate cancer tissue specimens. Using SPECS gene microarray data sets, which were generated by Dr. Dan Mercola's group at UCI (GEO access numbers are GSE17951 and GSE08218), the total of 204 samples from 82 patients include 66 tumor samples with tumor content greater than 40%, 65 tumor-adjacent stroma samples, 28 far stroma samples and 45 normal prostate samples. The mRNA expression levels of interesting genes were normalized to the average expression levels of these genes from the normal prostate samples. Figure 8 shows that percentage of samples with WIF1 down-regulation progressively increased from far stroma to adjacent stroma and tumors. Strikingly, the levels of NRP2 mRNA are up-regulated in all tested adjacent stroma and most of tumor samples. Furthermore, the expression levels of NRP2 are inversely related to WIF1 mRNA levels. These results suggest that, for a large series of clinical samples, NRP2 may be co-upregulated in adjacent stroma and most tumor samples and be associated with down-regulation of WIF1 expression (Ps<0.05).

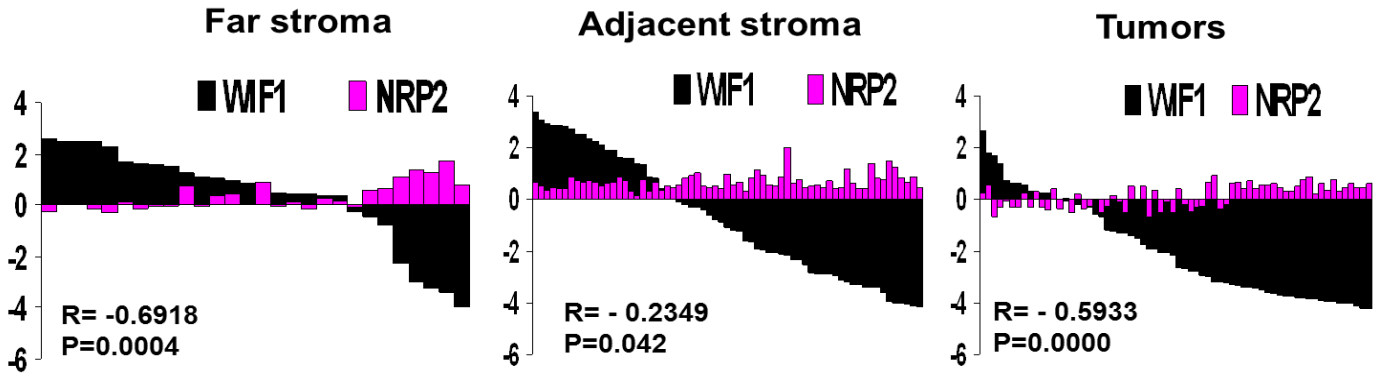



**Figure 8.** The expression levels of WIF1 and NRP2 and their correlations in prostate cancer tissues.

**2b.** To perform ChIP analysis for examination of TCF4/β-catenin binding to NRP2 promoter regions (months 8-12).

(1) LEF1 binds to NRP2 promoter regions with TCF/LEF1 responsive elements in vivo and inhibition of Wnt signaling by secreted antagonists inhibits the bindings. We performed a search for TCF/LEF1 responsive

elements in the promoter region of NRP-2 using GenBank and MatInspector software (Genomatix Software Inc., Ann Arbor, MI), an integrated bioinformatics software package for analyzing transcriptional regulation. In this database, a large library of predefined matrix descriptions for protein binding sites has been tested for accuracy. Five potential binding sites for TCF4 in the 5' flanking promoter region spanning from -3891 to +108 base pairs of NRP-2 gene were found: I (-2964 to-2958), II (-2426 to -2420), III (-979 to -973), IV(-647 to -631), and V (494 to 510) (Figure 9A). We then designed five sets of primers to cover this regions with TCF/LEF responsive elements. Using chromatin immuneprecipitation assay, we show that LEF1 binds to all five TCF/LEF responsive elements containing regions of NRP2 promoter (Figure 2). In addition, inhibition of Wnt signaling by expression secreted Wnt antagonists (WIF-1 and Frzb) significantly inhibited the binding of LEF1 to NRP2 promoter region in prostate cancer PC3 cells. Taken together, these results indicate that NRP2 is a Wnt target gene and regulated by secreted Wnt antagonists (WIF-1 and Frzb).

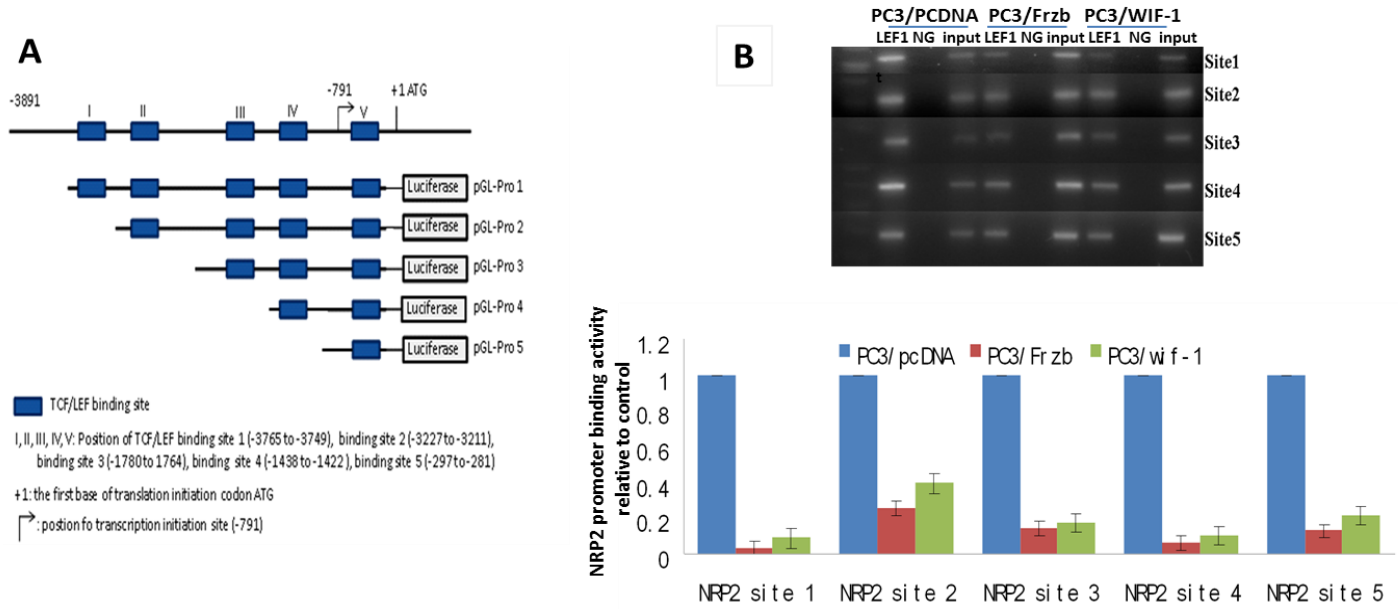



**Figure 9.** The promoter sequences of NRP2 contain multiple TCF/LEF binding sites and expression of secreted Wnt antagonists inhibit the binding of LEF1 to predicted TCF/LEF binding motifs in the NRP2 promoter in PC3 cells. (A) predicted position of TCF/LEF binding sites in the promoter of NRP2. (B) ChIP analysis of LEF1 binding to the NRP2 promoter. (C). Real-time PCR quantification of enrichment of LEF1 binding sequences.

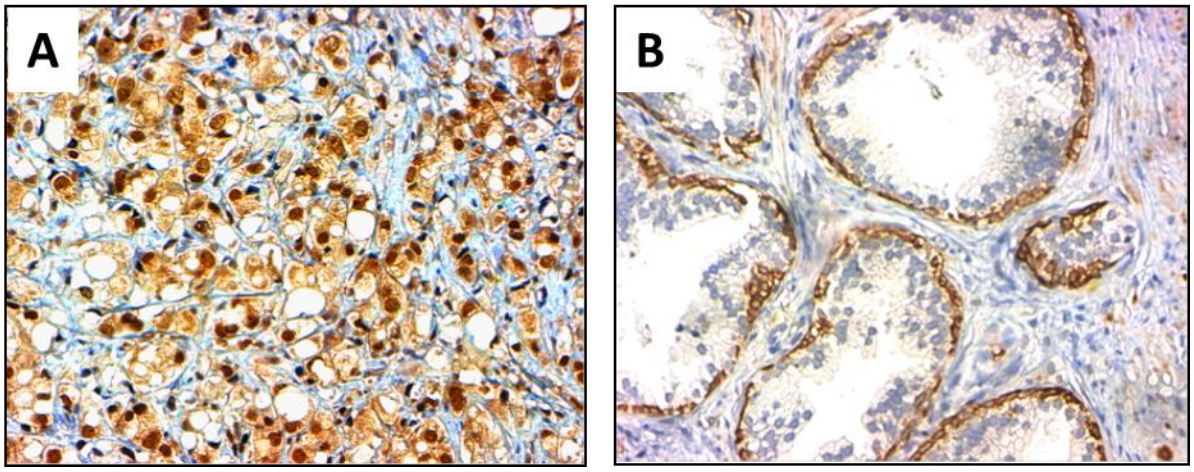
**Task 3. To determine whether the expression of NRP2 alone or in combination with VEGF receptors or c-Met will be associated with lymphatic vessel density, MVD, and WIF1 expression, and will be predictive for clinical and biochemical progression-free survivals of PCa patents, as well as for development of metastasis.**

**1a.** To Establish exclusion and inclusion criteria, make a questionnaire for data collection, establish and define data collection variables, contracture data base, collect pathological and clinical data (months 1-12).

(1) A database containing about 1000 prostatectomy patients from UCI medical center and VA long beach hospital with a follow-up for more than five years were established with SAS software. The database includes deidentified patients' information, preoperative PSA levels, follow-up PSA levels, age, diagnosis, pathological stages, tumor metastasis, surgical margin, seminal vesicle and others.

**1b.** To perform immunofluorescent labeling (IF) and quantification of biomarkers on TMAs, antibody optimization and staining, pathological annotation, imaging scanning and IF quantification, establish biomarker expression data base (months 1-18).

(1) LEF1 and NRP2 antibodies were validated by immunohistochemistry. Figure 10A &B shows that LEF positive stainings are located in the nucleus and cytoplasm of prostate cancer cells, and that NRP2 positive cells were found on the basal layer and stromal tissues and in the membrane and cytoplasm.




**LEF1 staining** **NRP2 staining**

**Figure 10** Immunohistochemistry analysis of LEF1 (**A**) and NRP2 (**B**) expression in prostate cancer tissues (X200 magnification)

## Key Research Accomplishments,

1. Construction of a WIF-1-human IgG expression construct and expression and purification of WIF-1-human IgG recombined protein.
2. The establishment of C4-2B/Luc and PC3/Luc reporter stable cell lines allows us to monitor prostate cancer growth and metastasis in vivo in orthotopic xenografts models.
3. Establishment of a prostate cancer orthotopic tumor xenograft mice model
4. Wnt stimulation by Wnt 3a and 5A and inhibition by WIF-1 and Frzb regulate the expression of NRP2.
5. Androgen deprivation increases Wnt signaling via induction of TCF4/LEF1 expression leading NRP2 up-regulation.
6. NRP2 promoter region contains multiple TCF/LEF1 responsive elements; Wnt stimulation increases NRP2 promoter activity; and LEF1 binds to the promoter region of NRP2 in vivo in prostate cancer PC3 cells
7. Secreted Wnt antagonists, WIF-1 and Frzb, inhibit the binding of LEF1 to the promoter of NRP2.
8. WIF-1 expression is progressively down-regulated in prostate cancer compared to far and adjacent stroma components in prostate cancer tissues, and inversely correlated with NRP2 expression.
9. Validation of LEF1 and NRP2 antibodies by immunohistochemistry.
10. Completion of constructing a prostatectomy follow-up database with collection of clinical and pathological information.

## Reportable Outcomes

1. Androgen deprivation increases Wnt signaling leading to NRP2 up-regulation.
2. Loss of a Wnt inhibitor, WIF-1, expression in prostate cancers was correlated with up-regulation of NRP2. WIF-1 is a potential tumor suppressor in human prostate cacner.
3. NRP2 is a Wnt target gene that is regulated by Wnt transcriptional factor LEF-1 in prostate cancer.
4. **Published Abstract.**

Shuman Liu, Zhenyu Jia, Zheng Sun, Xuesen Li, Yi Guo, Michael Lilly, Dan Mercola, Bang H. Hoang, **Xiaolin Zi.** Wnt signaling regulation neuropilin-2 (NRP2) expression and contributes to cancer cell invasiveness in castration-resistant prostate cancer (CRPC). In: Proceedings of the American Association for Cancer Research 103rd AACR Annual Meeting, 2012, March 31-April 4, 2012; Chicago, IL. Philadelphia (PA): AACR; Cancer Res 2012;72(8 Suppl): Abstract nr 440. doi:1538-7445. AM2012-440. Poster Session PO.TB04.o1 (Metastasis Biomarkers), Sunday, April 1, 2012.

## Conclusion:

Aberrant Wnt signaling has been associated with castration-resistant prostate cancer. In this study, we provide evidence that enhanced Wnt signaling by castration in prostate cancer result in an increased expression of tumor metastasis-related gene NRP2 leading to cancer progression. In addition, inhibition of Wnt signaling by a secreted Wnt antagonist, WIF-1, may be a viable approach in treatment of CRPC.

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